Neuronal and glial changes in white and grey matter of Multiple Sclerosis patients

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7

SUMMARY AND GENERAL DISCUSSION

SUMMARY

An overall pathological hallmark of MS is the loss of myelin and oligodendrocytes resulting in demyelination and lesion formation in the CNS. Although cases of MS patients suffering from GML were already described in the early 20th century,[478–481] research has focused mainly on WML, since conventional histochemical staining and imaging techniques were not, or only to a limited extent, able to visualize GML. Since the discovery of new imaging techniques, it is nowadays possible to visualize GML *in vivo* as well, which has led to a strong increase in research of this type of lesion.[482] WML can be categorized based on their pathological profile. The presence of infiltrated immune cells and activated glial cells are characteristic for active WML. These immune cells can still be found at the border of the lesions and not in the center of the lesion when the lesion becomes chronic active. Inactive lesions are characterized by a relative absence of infiltrated immune cells and activated microglia, while hypertrophic astrocytes fill the lesion and form a scar.[303–305] Interestingly, WML and GML differ in their cellular profile. WML show BBB damage and influx of immune cells.[303,304,354] Yet, BBB damage is not evident in GML[325] and influx of immune cells is significantly less compared to WML.[47,49,396] Therefore, it might be that, although demyelinating lesions occur in WM and GM of MS patients, the formation of WML and GML has a distinct underlying pathogenic mechanism and, consequently, development of novel therapies should take this into consideration. Currently available therapies for MS focus on the prevention of the infiltration of immune cells or dampening of the immune response in the CNS. However, these therapies do not stop the progressive phase of MS which involves mostly neurodegeneration in GML. Thus, it is of utmost importance to further characterize WML and GML and determine similarities and differences between these types of lesions. This may lead to a further understanding of the pathogenesis of WML and GML and lead to other or additional targets for therapy. The process of infiltration of immune cells through the BBB involves many molecules and receptors, including glial-derived factors such as chemokines.[365] The activation of microglia and astrocytes is a crucial and early event in the pathogenesis of both WML and GML formation.[49–53] However, GML present with less glial cells compared to WML, which questions whether differences between WM and GM glial cells contribute to the observed pathological cellular difference between WML and GML.

In an attempt to further characterize WML and GML pathology, we focused on hippocampal pathology for two different reasons. First, the hippocampus harbours both WM as well as GM, which makes this brain structure suitable to study WML and GML differences. Secondly, the hippocampus plays a pivotal role in the processes of learning and memory. MS patients often suffer from cognitive problems, e.g. memory problems,[20,189,190,192] and accumulating evidence suggests that hippocampal lesions are the underlying cause. Indeed, the hippocampus is often affected in MS patients[141–146] and hippocampal lesions correlate with memory deficits in these patients.[142] The major neurotransmitters involved in hippocampal learning and memory are glutamate, Ach and GABA. Disturbed glutamate signaling has already been described

in MS.[141,160] However, the status of cholinergic and GABAergic neurotransmission in MS patients is not yet known.

Hence, the two aims of the studies described in this thesis were:

1) **to determine the glial cell responsiveness in GM and WM of MS patients.**
2) **to identify changes in cholinergic and GABAergic neurotransmitter systems in the hippocampus of MS patients.**

In this final chapter, our main findings are summarized and discussed. In addition, directions for future research are given.

In **chapter 2**, we reviewed the literature to identify possible underlying mechanism(s) of pathological differences between WML and GML. First, the observed paucity of infiltrated immune cells in GML compared to WML might be, partly, due to the age of the lesions. Research to identify cells present in these types of lesions depends mostly on immunohistochemistry on *post mortem* tissue. Since analysis of biopsy material of MS patients did show infiltrated immune cells in GML,[217,294] it might be that these immune cells are present during the very early stage of GML formation.

Alternatively, differences between WML and GML can be the result of the local environment. While the GM is mainly composed of neurons and GM astrocytes and microglia, the WM harbours myelin forming oligodendrocytes, WM astrocytes and WM microglia. Neurons express several immune dampening molecules, e.g. CD200, CD47, ICAM-5 and CX3CL1. In addition, neurotransmitters that are released by neurons also modulate the immune response. Although the effects of glutamate and acetylcholine release on the immune response are dual, GABA is known for its immunosuppressive effects. Since GABA levels have been described to be higher in healthy GM compared to WM, this could contribute to the immunosuppressive environment of the GM. In addition, during MS, immune cells are directed against myelin and myelin breakdown and oligodendrocyte apoptosis occurs. The myelin debris that is left induces an immune response. Therefore, the immune response in GML could be less compared to WML due to lower levels of myelin debris in GML compared to WML.

Another difference between WM and GM that might account for the observed paucity of activated and infiltrated immune cells in GML is that BBB damage has been observed in WML, leading to infiltrating leukocytes in the parenchyma. Although there is no evidence for a physiological or anatomical difference in BBB in WM compared to the BBB in the GM, BBB damage has not been shown in GML.

Several lines of evidence indicate different reactivity of microglia and astrocytes in WM compared to GM. Although it remains unclear whether microglia and astrocytes from the WM are different types of cells compared to those found in the GM, it has been shown that glial cells in the WM express different molecules and can have a different phenotype than their GM counterparts. In humans, WM microglia have been described to be more

numerous compared to microglia in the GM.[326] In addition, WM microglia seem to have a stronger tendency to get activated and to be pro-inflammatory upon disturbed homeostasis.[483] Morphologically, WM astrocytes are described as with a fibrous-like morphology whereas GM astrocytes have a protoplasmic-like morphology.[318] In addition, in mice the presence of certain glial-derived chemokines involved in the migration of immune cells through the BBB is higher in WM compared to GM.[317] In this context, we analyzed the expression of the pro-inflammatory interleukin-1β (IL-1β) (**chapter 3**) and monocyte-chemotactic protein-1 (CCL2) (**chapter 4**) in WML and GML.

IL-1β is an important pro-inflammatory cytokine, being the principal driver of immune responses in the CNS.[62–64] IL-1β has been described to be involved in many processes during WML formation, e.g. oligodendrocyte apoptosis[97] and leukocyte migration.[68] We questioned whether IL-1β is also present in GML, in addition to WML. For this purpose, we analyzed the presence of IL-1β and its naturally occurring anti-inflammatory counterpart, the IL-1β receptor antagonist (IL-1ra) in the brain and spinal cord of cr-EAE rats. This experimental model mimics inflammatory pathology of MS and enabled us to analyze the presence of IL-1β and IL-1ra throughout the whole brain during the early phases of demyelination (**chapter 3**). We demonstrated that IL-1β and IL-1ra are present in GML, in addition to WML. This increase was strongest during the early phases of cr-EAE and suggests the involvement of IL-1β in both WML and GML formation, which was not prevented by the presence of endogenous IL-1ra.

CCL2 has been described extensively with regard to its ability to attract leukocytes across the BBB. A discrepancy between WM and GM expression of CCL2 and its receptor CCR2 may, therefore, play an important role in the pathological cellular difference observed between WML and GML. This hypothesis is corroborated by the findings described in **chapter 4**. The expression of CCL2 and CCR2 was analyzed in WML and GML of *post mortem* hippocampal tissue of MS patients. First, CCL2 en CCR2 mRNA levels were determined in hippocampi of healthy control subjects and myelinated and demyelinated hippocampi of MS patients. A significant upregulation of CCL2 and CCR2 mRNA was observed in demyelinated hippocampi compared to control hippocampi and myelinated hippocampi of MS patients. Immunohistochemistry for CCL2 and CCR2 was used to identify the spatial and cellular expression of CCL2 and CCR2 in active and inactive hippocampal lesions. Interestingly, CCL2 was barely detected in hippocampal GM, but was slightly increased in inactive hippocampal WML, and significantly upregulated in active hippocampal WML. Similarly, CCR2 was significantly increased in active hippocampal WML. However, in contrast to our findings on CCL2, CCR2 was also significantly upregulated in active GM hippocampal lesions. However, the number of CCR2 expressing cells was significantly higher in hippocampal WM compared to GM. The higher expression of CCL2 and CCR2 in hippocampal WML and low expression of CCL2 in GML could contribute to the observed difference in the number of infiltrating immune cells between WML and GML.

More than 50% of MS patients suffer from cognitive deficits, among which problems with memory is among the most frequently reported.[20] Hippocampal lesions have been linked to memory deficits in MS patients. The three major neurotransmitter systems involved in learning and memory are glutamate, Ach and GABA. Previous studies have already shown disturbed glutamatergic neurotransmission in MS. However, it is unknown whether cholinergic and GABA-ergic neurotransmission are affected in the hippocampus of MS patients.

The neurotransmitter Ach is of importance for learning and memory. The enzyme responsible for Ach synthesis is ChaT, while the enzyme AchE hydrolyzes Ach thereby reducing its concentration in the synaptic cleft. In chapter 5, ChaT and AchE activity and protein expression were examined in hippocampi of MS patients compared to hippocampi of patients with AD and control hippocampi. A severe decrease in ChaT and AchE activity in the hippocampus had already been established in AD, a neurodegenerative disease characterized by severe memory loss.[484] We found that ChaT activity and protein expression was significantly reduced in hippocampi of MS patients compared to control hippocampi. This reduction was comparable to the reduction observed in hippocampi of AD patients. Interestingly, AchE activity and protein levels remained unchanged in MS patients compared to controls, while these were significantly reduced in AD patients. These results suggest that in hippocampi of MS patients there is a reduced production of Ach, while the clearance of Ach from the synaptic cleft remains unchanged resulting in lower levels of Ach compared to controls. Therefore, treatment of cognitive decline in MS patients could possibly benefit from pharmacological inhibition of AchE activity.

In chapter 6, we examined PV and GAD67 expression, which are involved in GABAergic neurotransmission. PV represents a subgroup of GABAergic interneurons and GAD67 is an enzyme that converts glutamate into GABA, and is considered a marker for GABA. GABAergic neurotransmission is essential for optimal memory function.[186,187] The number of PV positive interneurons did not significantly differ between MS patients and controls subjects. Interestingly, we observed that the number of GAD67 positive interneurons significantly increased in and around active CA1 lesions of MS patients. In addition, GAD67 positive astrocytes were significantly more numerous in hippocampal WML than GML of MS patients and the level of GAD67 immunoreactivity in astrocytes is significantly increased in MS patients with active hippocampal lesions, and relates to self-reported cognitive impairment. Thus, this suggests that in MS patients GABAergic changes occur in neurons and astrocytes of which the latter possibly contributes to cognitive impairment.

GENERAL DISCUSSION

The pathology of GML in MS has attracted more research attention in the last decade. The clinical relevance of GML underlines the importance to understand more of the pathogenesis of GML in comparison to WML. Although both WML as well as GML are characterized by demyelination, their cellular pathology differs, i.e. infiltrating immune cells in WML outnumber those in GML. A possible underlying mechanism that could contribute to

this difference is differences between glial cells residing in WM versus GM. Therefore, we investigated glial cell responsiveness in GM and WM of MS patients.

IL-1β in cr-EAE

In **chapter 3** we described the expression of IL-1β and IL-1ra in WML and GML of rats suffering from cr-EAE. Our finding is in accordance with the results of previous studies, showing increased IL-1β in WML in EAE[71,218] and MS patients.[69,230] Since our study demonstrated that IL-1β is also present in GML, we indicated that IL-1β is a putative therapeutic target, not only to treat WML related deficits but also neurological impairments that are the result of GML.

Although we only detected single small demyelinated areas, the presence of lipid-laden macrophages and/or microglial cells in areas with decreased PLP staining indicated an ongoing demyelination process in WML and GML. This suggests that cr-EAE mimics the very early stages of lesion formation. Since IL-1β is known for its role during the initiation of WML formation,[83–86] our finding that IL-1β is simultaneously present in GM as well as WM suggests a comparable role for IL-1β in WML and GML formation. In addition, infiltration of $CD3^+$ T-cells was very limited and IL-1β expression was almost exclusively restricted to microglia and/or infiltrated macrophages. This indicates that microglia and/or macrophages are crucial in the development of both WML and GML in cr-EAE and is in accordance with previous studies showing that microglia activation precedes the infiltration of immune cells in EAE.[374,375] However, the exact cellular source of IL-1β warrants further study. Recently, infiltrating monocytes and resident microglia were compared regarding their expression of pro-inflammatory factors, e.g. IL-1β, when extracted during EAE and analyzed using a fluorescence-activated cell sorting (FACS)-based method. It was shown that infiltrating monocytes, and not microglia, were the major source of IL-1β.[485] In addition, another study using serial block-face scanning electron microscopy showed a more detrimental role for infiltrating macrophages during EAE while microglia seemed to have a more favorable role by clearing debris.[486] However, both studies did not distinguish between WML and GML. We observed morphological differences between CD68 positive microglia and/or macrophages, i.e. ramified CD68 positive cells in the GM versus amoeboid CD68 positive cells in WM. Therefore, further studies using FACS-analysis or electron microscopy of GM derived microglia and macrophages might shed more light on determining the cellular source of IL-1β in GML during cr-EAE.

Although the cr-EAE animal model we used in our study is very well suited to identify the involvement of IL-1β during the early phases of GML formation, in addition to WML formation, the regions where lesions occurred were mostly restricted to areas near ventricles, where the choroid plexus is located, and veins. Therefore, it might be that the IL-1β inducing signal is present in the choroid plexus or endothelial cells. Indeed, a significant increase of various cytokines, T-cell activation markers and chemokines was observed in different compartments of the choroid plexus, e.g. the stroma and the epithelial cells, during the early phase of MOG-induced EAE.[232] In addition, in MS patients the occurrence of cortical lesions

is related to the location of the principal cortical veins.[487] Moreover, the location of the lesions in our study on cr-EAE might also explain the absence of IL-1β within the hippocampus of cr-EAE. Areas that bordered the hippocampus, e.g. the habenula and the stria medullaris, did show IL-1β expression and are directly bordering the ventricles. In contrast, MOG induced EAE in mice does show hippocampal lesions containing IL-1β expressing monocytes and/or macrophages.[236] This emphasizes the importance to study the presence of IL-1β in GML of other MS animal models and in MS patients.

The absence of IL-1β in the hippocampus we observed in our study does not necessarily mean that the hippocampus is not affected by the induction of cr-EAE. Lesions within brain areas that are part of the in- and output pathways of the hippocampus, e.g. the habenula or the fimbria, might lead to disturbed signaling to or from the hippocampus. In this way, IL-1β might indirectly have an effect on hippocampal functioning.

Thus, WML and GML formation in cr-EAE involves IL-1β expression. This pro-inflammatory cytokine may hold promise as a putative therapeutic target, not only to treat WML related deficits but also neurological impairments that are the result of GML. Cr-EAE mimics relevant clinical and pathological characteristics of RR-MS and is therefore of value to study cellular mechanisms of demyelination. The use of this experimental model for MS enabled us to systematically investigate the expression of IL-1β and IL-1ra throughout the whole CNS. However, cr-EAE mimics inflammatory mechanisms during the early phases of demyelination and studying IL-1β and IL-1ra expression in brain tissue of MS patients remains of unique value and cannot just be replaced by studies in EAE. While our study emphasizes the overlap between WML and GML with respect to the expression of IL-1β and IL-1ra, the results do not explain the observed pathological difference between WML and GML. Therefore, other inflammatory factors that are known to be involved in the process of immune cell infiltration in MS should be studied. Interestingly, the expression of IL-1β is known to induce the expression of various inflammatory factors and chemokines, e.g. CCL2,[102] which is known for its function as an attractor of immune cells through the BBB in WML.

CCL2 and CCR2 are differentially expressed in WML and GML

Although IL-1β expression did not differ between WML and GML in cr-EAE rats, levels of CCL2 and CCR2 were significantly different in the WML and GML in the hippocampus of MS patients (**chapter 4**). Despite the fact that levels of CCL2 were virtually absent, CCR2 levels were significantly increased in active WML as well as GML. The significant increase of GM-derived microglia proliferation upon treatment with CCL2 suggests that CCL2-CCR2 interaction in GML might lead to a different outcome compared to WML, i.e. microglia proliferation versus infiltration of immune cells.

The virtual absence of CCL2 expression in GML compared to WML might be due to the fact that *post mortem* tissue is mostly available of MS patients in the progressive phase of the disease. Although CCL2 is then still present in active WML, we cannot exclude that

CCL2 had been present in GML in an earlier stage of the disease. *Post mortem* tissue of patients in an early stage MS is very limited, as MS patients age mostly like healthy humans. However, biopsy material can sometimes be used, although limited as well. Interestingly, it has been shown that in biopsy GML, in the early phase of MS, infiltrating immune cells were present.[217,294] Therefore, we cannot exclude the possibility that CCL2 is still important in GML to attract immune cells during the initial phase of GML formation. In addition, it might be that inflammation in GML is quickly resolved due to intrinsic characteristics of the GM, i.e. the tendency of GM to express anti-inflammatory factors or immune dampening factors. Alternatively, the difference in CCL2 expression between WM and GM might be the consequence of functional differences between WM and GM astrocytes. Morphologically, WM astrocytes are described as with a fibrous-like morphology whereas GM astrocytes have a protoplasmic-like morphology.[318] Only little evidence is available on molecular differences between fibrous and protoplasmic astrocytes and it remains unclear whether WM and GM astrocytes are functionally different. Since axonal and/or cellular damage upon demyelination can induce increased levels of ATP in the extracellular space,[307] we observed that bz-ATP, a potent analogue of ATP, induced expression of CCL2 which was significantly higher in WM-derived astrocytes than in GM-derived astrocytes. Therefore, it might be that WM astrocytes are intrinsically different from GM astrocytes, but this remains to be established unequivocally in future studies.

Although CCL2 is the principal driver of leukocyte migration, other chemokines, adhesion molecules and MMP's are also involved in this complex process. Interestingly, an increase in both CCL2 as well as CCL3 was observed in WML and GML of cuprizone treated mice[317] and genetic deletion of both chemokines resulted in a significant decrease in cortical demyelination.[488] This emphasizes the importance of chemokines in GML formation, without the influx of immune cells. Moreover, CCL2, and not CCL3, was found to be involved in relapsing EAE, while CCL3, and not CCL2, was linked to acute EAE,[489] suggesting a differential role for each of these chemokines. Therefore, the presence of other chemokines involved in leukocyte migration identified in WML of MS patients, e.g. CCL3,[299] should be investigated in GML as well.

Neurotransmitter changes in the hippocampus of MS patients

In addition to the fact that the hippocampus harbours WM and GM, this brain structure is of crucial importance to the process of learning and memory. Several excitatory and inhibitory neurotransmitters act together to induce LTP, which is the underlying mechanism of learning and memory. In addition to already studied characteristics of glutamate neurotransmission in MS, Ach and GABA are crucial neurotransmitters involved in learning and memory. Demyelination induced damage of Ach and GABA producing cells might affect Ach and GABA levels within the hippocampus of MS patients. Therefore, in this thesis we aimed at identifying changes in cholinergic and GABAergic neurotransmitter systems in the hippocampus.

Interestingly, in **chapter 5** we showed a significant reduction in ChaT activity and protein expression in the hippocampus of MS patients which occurred regardless of the local presence of lesions. This might be explained by the fact that the majority of the cholinergic input to the hippocampus comes from the medial septum and diagonal band of Broca.[166] Therefore, reduced ChaT activity is likely to be the consequence of decreased activation of cholinergic neurons within the forebrain, leading to decreased protein expression and activity of ChaT. Future studies should therefore incorporate these forebrain structures into analyses on cholinergic neurotransmission in MS. In addition to reduced ChaT protein levels and activity, AchE protein levels and activity remained unaltered. The reduced ChaT activity together with unaltered AchE activity suggests that Ach levels are reduced in the hippocampus of MS patients. Since Ach is one of the crucial neurotransmitters in learning and memory, a decrease in cholinergic input in the hippocampus might contribute to deficits in learning and memory. Interestingly, within the pyramidal cell layer, i.e. the cornu ammonis, of the hippocampus, PV positive GABAergic interneurons are located.[490] Excitatory neurons within the hippocampus are the primary target of this type of interneuron. In **chapter 6** we showed that the number of PV positive neurons is not changed in MS patients compared to healthy controls. Of interest, PV positive interneurons express muscarinic Ach receptors (mAChR), activation of which leads to decreased GABA release within the CA1 region.[491] Therefore, the decreased Ach levels in MS hippocampi we observed in **chapter 5** could lead to reduced inhibition of GABA release.

Although PV positive interneurons are the most numerous subtype of GABAergic interneurons in the hippocampus,[459] and have been described to play a crucial role in hippocampus dependent memory,[460] other GABAergic interneurons, e.g. calbindin (CB) and calretinin (CR) positive interneurons, located within the stratum radiatum mainly affect the integration of excitatory input.[492] Moreover, cholecystokinin (CKK) positive interneurons have been shown to be more prone to Ach excitation due to their higher density of nAchr and mAchR compared to PV positive interneurons.[492,493] Therefore, future studies should expand our knowledge on neurotransmission in MS, by investigating these other types of GABAergic interneurons as well.

Interestingly, in **chapter 6** we did observe that significant changes in GAD67 are present in MS patients, i.e. the number of GAD67 positive neurons was significantly increased in and around active CA1 lesions of MS patients. This suggests that GABA levels are increased in and around active lesions. In addition, in hippocampal WM of MS with active lesions we detected a significant increase in the density of GAD67 positive astrocytes. Together, this suggests an increase in GABA levels in active lesions in the hippocampus of MS patients. This might be a response to excessive glutamate levels as a consequence of neuronal and axonal damage in hippocampal lesions,[307] or it might be induced to dampen the immune response.[476] Alternatively, increased GABA levels may be the result of reduced inhibition by Ach. Interestingly, this increase in GAD67^{+} astrocytic density correlated with self reported cognitive decline. Therefore, these findings reveal a specific target for therapeutic interventions

to improve cognitive function in MS patients.

In contrast to our findings on globally decreased ChaT levels in **chapter 5**, increased GAD67 immunoreactivity was only observed within hippocampal lesions, suggesting that more GABA is present in and around CA1 lesions within the hippocampus. Interestingly, another study showed decreased glutamate levels in the hippocampus of MS patients.[160] Therefore, it might be that increased GABA levels together with the reduced levels of Ach, which is known for its glutamate potentiating effect, in MS hippocampi result in the inhibition and/or reduced induction of glutamate production. Although inhibition of glutamate production might be useful to prevent or counteract glutamate excitoxicity, glutamate inhibition might be devastating for memory performance since it is needed for LTP.[165] In addition, a reduction in mRNA encoding NMDA and AMPA receptors has been described in demyelinated MS hippocampi,[141] which might also contribute to disturbed glutamate action and subsequent reduced memory function.

Although we found a significant increase in neuronal GAD67 immunoreactivity, it is of importance to note that astrocytic GAD67 contributed largely to the observed increase in GAD67 staining in WML. This might point towards an immune modulating effect of GABA. GABA-A receptors present on macrophages mediate the decreased inflammatory response initiated by these cells upon interaction with GABA.[476,477] Moreover, it has been reported that EAE onset is delayed and severity decreased after increased GABAergic activity.

Inflammatory factors and memory

Neurotransmitter signalling can be modulated by inflammatory factors (reviewed in: [494]). In fact, physiological levels of pro- and anti-inflammatory mediators are essential for proper neurotransmission and LTP to occur. For example, a low level of IL-1 is required for the initiation and maintenance of LTP in vitro.[495] In addition, mice that have a deletion of the IL-1 receptor type I gene or overexpress IL-1ra show deficits in hippocampus-dependent learning and memory.[496,497] Similar results have been observed concerning IL-6 and TNF-α.[494] Moreover, expression of neurotrophic factors such as brain-derived neurotrophic factor (BDNF)[498] and nerve growth factor (NGF)[499] is needed for learning and memory. However, when homeostasis within the brain is disturbed by (chronic) neuroinflammation, neurodegeneration or an infectious disease, an increase in pro-inflammatory factors occurs which can be detrimental to memory functioning. Indeed, lipopolysaccharide (LPS) induced upregulation of e.g. IL-1β and TNF-α levels resulted in deficits in learning and memory.[500]

The underlying mechanisms of the detrimental effects of enhanced levels of pro-inflammatory cytokines on memory range from the modulation of neurotransmission to the suppression of neurotrophic factor production. Glutamatergic[474] and GABAergic[501] neurotransmission is increased and reduced, respectively, by expression of IL-1β. Similarly, acetylcholine release from the hippocampus is reduced by IL-1β.[270,271] In addition, expression of pro-inflammatory factors such as IL-1β inhibit the expression of neurotrophic factors, e.g. BDNF.[502] Thus, neuroinflammation, as observed in MS patients, can directly or indirectly

affect memory functioning. Indeed, in MS patients, higher levels of IL-6 and lower levels of BDNF correlated with cognitive deficits observed in these patients.[503] Furthermore, a decrease in NGF was observed in EAE rats, which correlated with memory impairments.[124] Both resident immune cells as well as infiltrating leukocytes are able to induce the production of pro-inflammatory cytokines. Thus far, however, it remains to be determined whether inflammatory factors produced by resident glial cells can lead to memory impairment in MS or whether infiltrating leukocytes are of crucial importance in this process. Preliminary results from a study in cuprizone mice (described in the paragraph below) point towards an important role for infiltrating leukocytes in addition to activation of resident immune cells.

Demyelination, inflammatory factors and memory function

In MS, memory impairment is a frequently identified problem, which has been hypothesized to be caused by e.g. hippocampal demyelination. Hippocampal demyelination is, similar to other GML, characterized by a paucity of immune cells. To mimic the absence of leukocytes in the brain, but demyelination to occur, the cuprizone model can be used. This experimental animal model is characterized by severe demyelination of the corpus callosum without the influx of immune cells upon cuprizone treatment.[130,131] Recently, demyelination of the hippocampus has also been identified in the cuprizone mouse model.[504] To identify changes in hippocampal functioning in cuprizone mice, we tested the mice on their performance on the modified Barnes Maze (mBM). The materials and methods used in these studies are described in box 1.

BOX 1. MATERIALS AND METHODS

Modified Barnes Maze

The mBM is a validated tool to study hippocampus-dependent spatial memory in mice[505] and is adapted from the original Barnes Maze by adding extra escape holes that prevent mice from solving the task on the basis of a non-spatial, e.g. serial exploration strategy, which is considered to involve the striatum.[506] The mBM is a circular platform containing 44 holes, including one escape hole (Figure 1). Mice are placed in the middle of the maze and are trained to locate the escape hole using visual extra-maze cues on the distant walls as a guide, and being motivated to escape the platform by ventilator-produced wind blown over the platform. The time needed to locate the escape hole and number of errors is recorded. The number of errors is defined by the number of nose pokes into holes that are not the escape hole.

Behavioral experiment 1: Memory retention

Sixteen C57BL/6 (6-8 week old) were trained for 3 minutes on the mBM for 7 consecutive days, twice a day. After this mBM training, eight mice were kept on normal chow, while the other eight animals were fed with a diet containing 0.2% cuprizone (bis-

cyclohexanone oxaldihydrazone, Sigma-Aldrich Inc., St. Louis, MO, USA), for 5 weeks. During these 5 weeks of cuprizone treatment, cuprizone-treated mice and control mice were tested once a week, twice a day, on their performance on the mBM. After the 5th week and last test on the mBM, mice were sacrificed and their brains were removed and immediately fixed in 4% PFA overnight. Immunohistochemical staining for myelin basic protein (MBP) (1:500; Boehringer) was performed on 5 μm coronal paraffinized sections.

Behavioral experiment 2: Memory acquisition

Fourteen C57BL/6 (6-8 week old) were given a diet containing 0.2% cuprizone for 3 weeks during which they were trained on the mBM. Training on the mBM started after 2 weeks of cuprizone treatment. The results were compared to 12 control mice which were fed for 3 weeks with normal chow. An additional fourteen mice were fed the 0.2% cuprizone diet for 5 weeks during which they were also trained on the mBM, starting after 4 weeks of cuprizone treatment, compared to 12 control mice. All mice were trained for 3 minutes, twice a day, for 6 consecutive days on the mBM. At the end of the behavioural task, the mice were sacrificed and their brains were removed and immediately fixed in 4% PFA overnight. Immunohistochemical staining for myelin basic protein (MBP) (1:500; Boehringer) was performed on 5 μm coronal paraffinized sections.

Semiquantitative RT-PCR (q-PCR)

In another experiment, C57BL/6 (6-8 week old) mice were given a diet containing 0.2% cuprizone for 1, 2, 3, 4 or 5 weeks (n=6/group). In addition, two groups of mice (n=6/group) were given cuprizone for 5 weeks after which they were given normal chow for 1 or 2 weeks, respectively. Terminating the cuprizone diet stopped the demyelination process and induced remyelination. A control group of mice was given only normal chow for 5 weeks. The hippocampi of these mice were processed for RT-PCR for CCL2 and IL-1β relative to the level of the housekeeping gene hypoxanthine phosphoribosyl transferase (HPRT). RNA extraction, cDNA production and RT-PCR was performed using previously published methods.[397]

Details of the primer sequences are as follows:
CCL2 forward primer: 5'-CAGCCAGATGCAGTTAACGC-3'
CCL2 reverse primer: 5'-GCCTACTCATTGGGATCATCTTG-3'
IL-1β forward primer: 5'-AAAGAAGAAGATGGAAAAGCGGTT-3'
IL-1β reverse primer: 5'-GGGAACTGTGCAGACTCAAACTC-3'
HPRT1 forward primer: 5'-CTCATGGACTGATTATGGACAGGAC-3'
HPRT1 reverse primer: 5'-GCAGGTCAGCAAAGAACTTATAGCC-3'

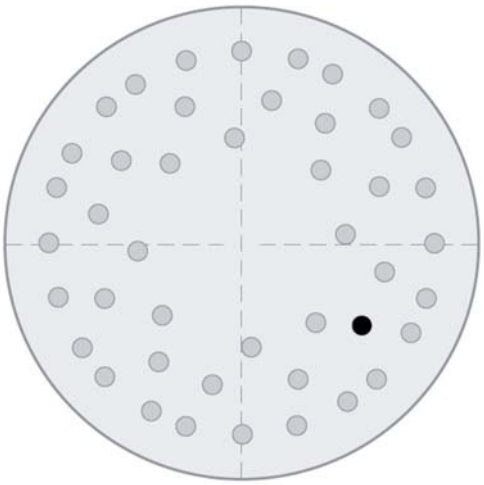

Figure 1. Schematic representation of the modified Barnes Maze. The modified Barnes Maze (mBM) is used as a tool to study spatial hippocampus dependent memory. The mBM consists of 44 holes, arranged symmetrically within each quadrant. Mice are trained for seven days to find the escape hole, indicated by the black colour. (Adapted from:[505]).

The first behavioral experiment indicated that cuprizone-treated mice performed less well on the mBM compared to control mice. Although these results did not reach significance ($F = 3.4$, $p = 0.09$) (Figure 2), there was a clear tendency that the cuprizone-treated mice made more errors before retrieving the location of the escape hole. Immunohistochemical analysis revealed severe demyelination of the hippocampus after 5 weeks of cuprizone treatment, similar to our immunohistochemical results found in the second behavioural experiment (Figure 4). In addition, the second behavioral experiment showed that cuprizone-treated and control mice performed equally well in learning to find the escape hole ($F = 0.23$, $p = 0.6$), and there was no difference between animals fed with cuprizone for 3 or 5 weeks ($F = 0.23$, $p = 0.6$). Thus their spatial memory acquirement was not affected by the cuprizone diet, i.e. demyelination of the hippocampus (Figure 3). Upon immunohistochemical analysis the mice brains showed indeed some hippocampal demyelination in the stratum radiatum after 3 weeks of cuprizone treatment which was more extensive after 5 weeks of cuprizone treatment (Figure 4).

Interestingly, the qPCR experiment demonstrated that IL-1β mRNA levels significantly increased during cuprizone treatment (ANOVA, $F=8.42$, $p < 0.001$), already after 1 week of cuprizone treatment (LSD, $p < 0.001$) and increased until 5 weeks of cuprizone treatment. After 1 and 2 weeks of remyelination, IL-1β expression decreased significantly compared to the group of mice that was treated with cuprizone for five weeks (LSD, $p < 0.001$). However, IL-1β mRNA was still significantly higher during remyelination compared to control mice (LSD, $p < 0.05$) (Figure 5A). Also CCL2 mRNA levels were significantly increased after cuprizone treatment (ANOVA, $F=23.27$, $p < 0.001$). CCL2 mRNA levels were highest after 1 week of cuprizone treatment (LSD, $p < 0.01$ compared to all groups, except 4 weeks of cuprizone treatment). During the remyelination phase, CCL2 decreased significantly

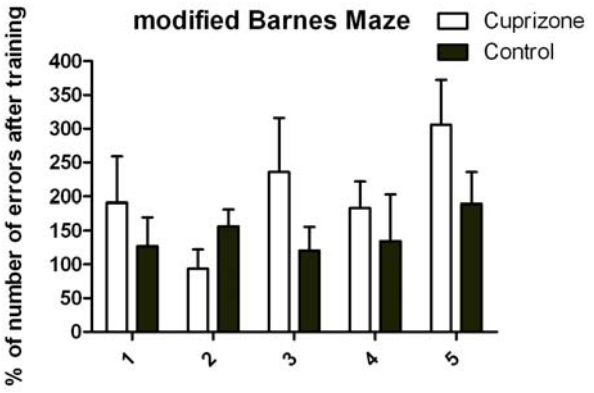

Figure 2. Memory retrieval. Before cuprizone treatment, mice were trained on the modified Barnes Maze. After training, cuprizone treatment started and control mice (n=8), fed with normal chow, and cuprizone treated mice (n=8), fed with chow mixed with 0.2% cuprizone, were tested once a week on the mBM. The number of errors, i.e. nose pokes in holes that are not the target hole, was measured and the percentage was calculated based on the number of errors during the last training for each mouse. Control mice seem to perform better on this task compared to cuprizone-treated mice, although the difference failed to reach statistical significance.

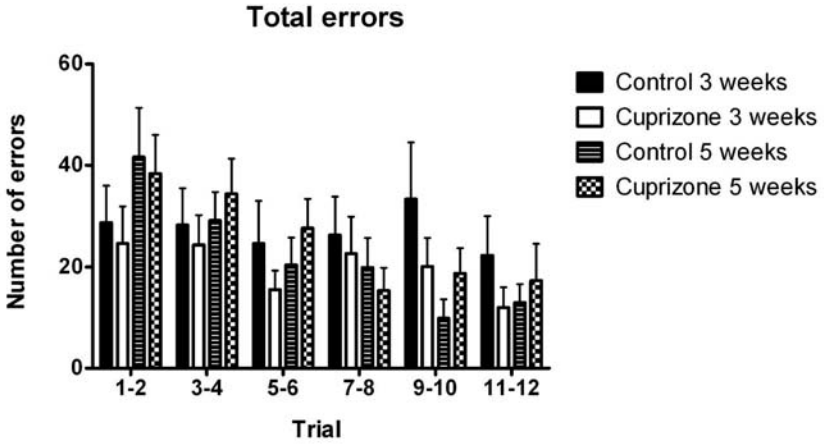

Figure 3. Memory acquisition. Control and cuprizone-treated mice were trained on the mBM. One group of mice was trained starting after 2 weeks of cuprizone treatment (n=14) compared to a control group (n=12) that was given normal chow for 3 weeks. Another group of mice was trained starting after 4 weeks of cuprizone treatment (n=14) compared to a control group (n=12) that was given normal chow for 5 weeks. The number of errors, i.e. nose pokes in holes that are not the target hole, was measured. No difference between the groups was detected.

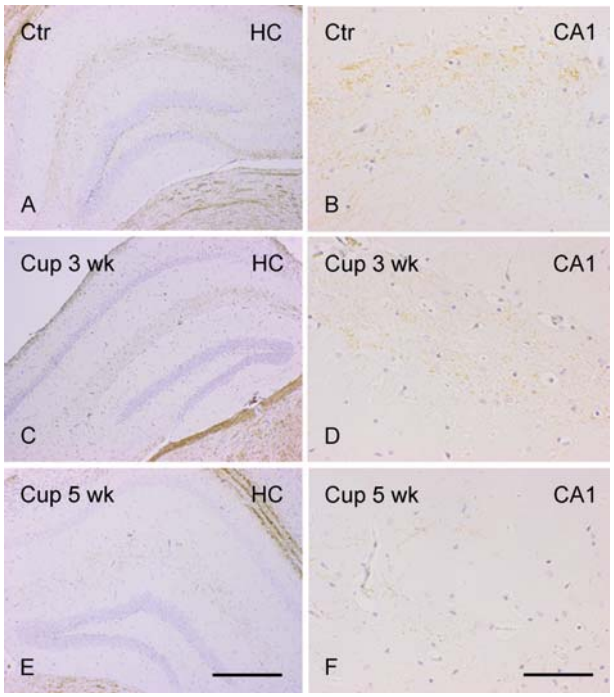

Figure 4.
Hippocampal demyelination in cuprizone-treated mice. Myelination of the hippocampus was visualized using an mylin basic protein (MBP) antibody. (**A**, **B**) In control mice, MBP immunoreactivity (ir) was visible within the stratum radiatum of the CA1 area. (**C**, **D**) After three weeks of cuprizone treatment the MBP ir was reduced, and (**E**, **F**) almost completely absent after five weeks of cuprizone treatment. **B**, **D** and **F** are higher magnifications of the stratum radiatium in the CA1 area of **A**, **C** and **E**, respectively. Scale bar (**A**, **C**, **E**) = 500 μm, scale bar (**B**, **D**, **F**) 100 μm.

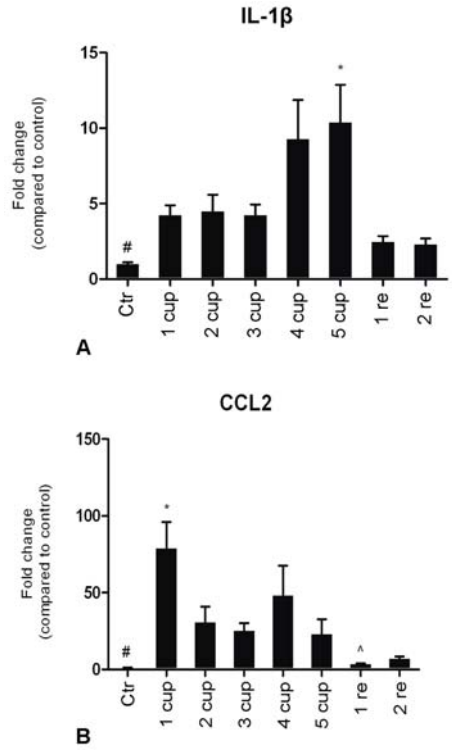

Figure 5.IL-1β and CCL2 mRNA in the hippocampus of cuprizone-treated mice.
(**A**) IL-1β mRNA was significantly increased in the hippocampus of cuprizone-treated mice already after 1 week of treatment. #$p < 0.05$ compared to all other groups; *$p < 0.05$ compared to all other groups, except 4 weeks of cuprizone treatment. (**B**) CCL2 mRNA was significantly increased in the hippocampus of cuprizone-treated mice with a maximum after 1 week of cuprizone treatment. #$p < 0.001$ compared to all other groups; *$p < 0.01$ compared to all other groups, except 4 weeks of cuprizone treatment. ^$p < 0.05$ compared to all other groups, except 2 weeks of remyelination.
Abbreviations: cup: cuprizone, re: remyelination

compared to demyelinating cuprizone mice (LSD, $p < 0.001$ compared to all groups, except 2 weeks of remyelination). However, CCL2 mRNA levels remained higher during remyelination compared to control mice (Figure 5B).

These unpublished data suggest that demyelination and activation of resident immune cells without the influx of immune cells can contribute to, but are not the major driver of, changes in hippocampus-dependent spatial memory. The observed hippocampal increase in IL-1β and CCL2 mRNA emphasizes the involvement of these pro-inflammatory cytokines already in early hippocampal demyelination. Although the cuprizone mouse model is known for the absence of infiltrating immune cells across the BBB, we observed a significant increase in CCL2 mRNA in the hippocampus of cuprizone-treated mice. Such infiltrating immune cells might be required to induce functional hippocampal problems. Alternatively, hippocampal functioning of cuprizone-treated mice is, albeit only mildly, affected, but the mBM might not be suitable to detect such subtle differences in hippocampal functioning and/or the cuprizone treatment is too mild to firmly induce grey matter demyelination. These suggestions are supported by the recent observation that mice treated with cuprizone for 12 weeks in combination with rapamycin induced more severe hippocampal demyelination compared to mice treated with cuprizone only. These cuprizone/rapamyin treated mice performed significantly worse on a hippocampus-dependent spatial tasks, i.e. the Morris water maze.[301]

CONCLUSIONS AND SUGGESTIONS FOR FUTURE RESEARCH

To conclude, we have shown that the expression of IL-1β, IL-1ra, CCL2 and CCR2 appear in WML as well as GML. However, IL-1β expression in WML and GML was similar, while a discrepancy in CCL2 expression between WM and GM could contribute to the paucity of infiltrated leukocytes in GML. Thus, glial cell responsiveness in WM seems to differ from that in GM, in active MS lesions. Interestingly, the relative absence of infiltrating immune cells in GML does not prevent GM demyelination, which we also demonstrated in our cuprizone experiments where the BBB remains intact but cortical and hippocampal lesions occur.[130,131] One of the recent hypotheses on the pathogenesis of GML states that WML and GML formation starts from the inside-out, i.e. a local stimulus inside neurons or oligodendrocytes results in demyelination and neurodegeneration that subsequently induces a secondary inflammatory response that further boosts the pathology.[45,507] In that respect, the increased expression of IL-1β in WML and GML of cr-EAE mice could reflect a secondary response. Also in cuprizone-treated mice, the expression of IL-1β increases in early stage pathology and maybe the response to a local event in the CNS, irrespective of the presence of infiltrating cells.

IL-1β induces increased expression of the ATP receptor P2X7 on astrocytes,[508] which makes them more prone to react to ATP, with subsequent increased CCL2 production. Since we observed a significant difference in the level of CCL2 expression between WM and GM of MS patients, as well as an increased CCL2 expression in hippocampus of cuprizone-treated

mice, and IL-1β expression in WM and GM of cr-EAE rats, future studies should elaborate on differences between WM and GM astrocytes. WM and GM astrocytes are described to present with distinctive morphological features, i.e. WM astrocytes present with a fibrous-like morphology whereas GM astrocytes have a protoplasmic-like morphology.[318,509] In addition, molecular differences between WM and GM astrocytes, e.g. the differential expression of the gap junction protein Cx30 and the excitatory amino acid transporter EAAT2, have been described [319,510] However, molecular and functional differences between fibrous and protoplasmic astrocytes in the context of MS remains to be clarified, e.g. the astrocytic expression of purinergic receptors like P2X7. Moreover, we observed a significant increase in GAD67 in WM astrocytes, suggesting an increased GABAergic response in these cells which warrants further elucidation.

Under healthy conditions, there is a balance between glutamatergic, cholinergic and GABAergic neurotransmission facilitating learning and memory. However, an inflammatory trigger, e.g. IL-1β, might disturb this balance. Indeed, in the hippocampus of MS patients we observed changes in the cholinergic and GABAergic neurotransmitter systems. Ach release is affected by GABA and glutamate, due to the presence of GABA-A receptors and glutamate receptors on cholinergic neurons.[511] Conversely, α7 nAchR have been identified on glutamatergic axons terminals in the striatum and the neocortex of rats and humans, respectively. Activation of these AchRs mediated increased glutamate release.[512,513] In addition, glutamate can act as a substrate for GABA production and GABA is known for its inhibiting effect on glutamatergic neurotransmission.[158,159] Since the cholinergic, GABAergic and glutamatergic neurotransmitters influence each other, future studies should focus on the characterization of the balance between these neurotransmitter systems. For example, serial tissue sections could be stained immunohistochemically for cholinergic, GABAergic and glutamatergic markers. In addition, a micro-array study could be performed to identify which genes involved in these types of neurotransmission are differentially expressed in the hippocampus of MS patients compared to healthy control subjects.

Finally, in the process of learning and memory, other brain regions, besides the hippocampus, are involved, e.g. the perirhinal, the entorhinal, the parahippocampal and the prefrontal cortex.[155–157]Although clearly more brain areas need to be studied to fully elucidate the origin of cognitive dysfunction in MS patients, we nevertheless expect that the hippocampus will prove to be a central player due to the various anatomical connections with other brain areas. Importantly, though, to be able to identify cellular and molecular mechanisms underlying memory deficits in MS, there is an urgent need to systematically document cognitive dysfunction in MS patients. In addition, the type of cognitive dysfunction of MS patients needs to be described to be able to correlate these with observations in *post mortem* brain tissue of MS patients. Currently, due to the absence of documentation on the occurrence, the type and extend of cognitive problems, several studies that touched upon this subject were unable to draw firm conclusions concerning underlying cellular or molecular changes. For example, Dutta et al. 2011 extensively described and investigated a molecular

mechanism possibly underlying memory deficits in MS. However, *post mortem* tissue of MS patients was used of which no information on ante mortem memory function was available.[141] In addition, Geurts et al. 2007 showed that lesions within the hippocampus are related to cognitive decline in MS patients. However, no information on the exact type of cognitive decline was available or determined in these patients.[142] Recently, tools to measure cognitive impairment in MS patients have been reviewed,[514] and the Brief International Cognitive Assessment for Multiple Sclerosis (BICAMS) was recommended as a valuable tool for cognitive assessment.[515] However, it is to be recommended to use a more extensive neuropsychological testbattery like the minimal assessment of cognitive function in multiple sclerosis (MACFIMS),[427] which covers the five most affected cognitive domains of MS patients. The importance of the assessment of cognitive performance is underlined by recent results showing that cognitive dysfunction in MS patients is a good predictor of disease progression.[516]

In conclusion, this thesis suggests that GML formation shares a similar trigger compared to WML, as indicated by the presence of IL-1β in both WML as well as GML. In addition, we showed that CCL2 and CCR2 expression differs significantly between WML and GML, while both hippocampal WML and GML are characterized by demyelination, which may suggest that the mechanism of demyelination differs between WML and GML. Since our observations are restricted to *post mortem* hippocampal tissue of MS patients, we are unable to determine whether the initial inflammatory response differs between WML and GML. Although mixed WML and GML in *post mortem* brain tissue show a significant difference in the number and activation of infiltrating immune cells between these two types of lesions,[47,396] investigation of WML and GML in biopsy material revealed that infiltrating immune cells are present in early cortical GML.[217,294] Nevertheless, higher expression of CCL2 and CCR2 in WML compared to GML, might result in a different response to the initial inflammatory response in early lesion formation, i.e. more infiltration of immune cells in WML compared to GML. Interestingly, our studies on cuprizone treated mice, which lack infiltrating immune cells, did not show that hippocampus dependent spatial learning is affected after hippocampal demyelination. However, in *post mortem* human MS hippocampal tissue we found a correlation between the presence of GABAergic astrocytes in hippocampal WML and self reported cognitive dysfunction. This might indicate that glial cells contribute significantly to neurological dysfunction during demyelination.

Cognitive dysfunction present in MS patients puts a strong burden on their quality of life, even more than their physical contraints. Up till now, no treatment for cognitive problems exist. Therefore, cognitive dysfunction should be the topic of extensive future research. A correlation between GML and cognitive dysfunction has been reported, which directs the attention to the underlying mechanisms of GML formation. Since GML differ in some pathological aspects from WML, further characterization of these differences is warranted as this may implicate alternative therapeutical strategies for treating GML. In this thesis, we contributed to the characteristics of WML and GML differences and suggested a role

for WM and GM astroglial differences, i.e. differential expression of CCL2. Furthermore, we identified elements of neurotransmitter systems involved in learning and memory, which are affected in MS patients. Therefore, astrocytes as well as neurotransmitters could be a potential target for therapy. The diversity of astrocytic function, ranging from immunomodulation to neurotransmitter regulation, highlight the complexity of glia-neuron interaction which should gain more attention in the context of MS to be able to identify therapeurtic targets and the exact conditions and timing of therapeutic treatment.